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(57) Abstract

Eukaryotic folding complexes and subunits thereof are disclosed, having increased solubility and/or biological activity due to association with at least one folding complex or subunit thereof. Engineered hosts expressing recombinant or endogenous folding complexes are also disclosed, as well as methods for producing soluble and/or biologically active heterologous proteins or polypeptides.

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A CYTOPLASMIC CHAPERONIN AND METHODS OF MAKING AND USING IT

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to novel eukaryotic folding complexes, subunit polypeptides and fragments thereof and nucleic acids encoding therefor, which are useful for providing proteins or polypeptides having increased solubility and/or biological activity due to association with the folding complex or subunits thereof. The present invention is further related to a recombinant or engineered host expressing at least one introduced or amplified folding complex, as well as methods for producing soluble and/or biologically active heterologous proteins or polypeptides in recombinant and/or engineered hosts using such nucleic acids.

Description of the Background Art

Biotechnology has provided the ability to genetically engineer or modify hosts to produce heterologous proteins in large amounts using recombinant DNA technology.

However, a major unsolved problem in recombinant DNA technology is the solubility and biological activity of a recombinant protein when it is overexpressed in a host, especially a bacterial host or a yeast host. Typically, most eukaryotic proteins and even many heterologous prokaryotic proteins are produced in substantially insoluble and/or biologically inactive form in bacteria such as *Escherichia coli* (*E. coli*), accumulating in the bacterial cytoplasm as "inclusion bodies" (IBs). IBs consist mainly of a recombinant protein, commonly in the form of a fusion protein with a bacterial protein, plus non-reducible polymers of the protein. IBs can be as large as the bacterial cell itself, and may also contain the four subunits of RNA polymerase, some combination of one or more of the outer membrane proteins OmpC, OmpF and OmpA, 16S and 23S rRNA, and circular and nicked plasmid DNA (Hartley, D.L. et al., *Biochem. Soc. Transac.* 16:101-102 (1988)).

Solubilization of the desired protein from IBs typically requires use of strong chaotropic agents, such as

relatively high concentrations of urea or guanidinium HCl. The denatured protein must then be correctly refolded during, or after, removal of the denaturing agent. Successful performance of this refolding step has proven difficult, if not impossible, for larger proteins. (For purification of refractile or IB proteins, see Jones, U.S. Patent 4,512,922; Olson, U.S. Patent 4,518,526; Builder et al., U.S. Patents 4,511,502 and 4,620,948)..

Accordingly, proteins that are substantially insoluble when expressed in *E. coli*, such as, for example, antibodies, tissue plasminogen activator (t-PA) and Factor VIII, must be produced in eukaryotic cell cultures rather than in bacteria. Eukaryotic cell cultures, such as mammalian or yeast cell cultures, require a more complex and more costly operation than bacterial fermentation. However, even in yeast and mammalian cell culture systems, lack of solubility or biological activity may continue to be a problem. For example, when t-PA was produced in a yeast cell secretion system, the protein was predominantly membrane-associated (Hinnen, A. et al., In: *Yeast Genetic Engineering*, Barr, P.J. et al., Eds., Butterworths, 1990, pp 193-213). Factor VIII was also found to accumulate in the Golgi apparatus after induction of producer monkey cells (Sedivy, J.M., *Bio/Technology* 6:1192-1196 (1988)). While a protein begins its synthesis in the endoplasmic reticulum (ER), it is shuttled from one cellular compartment to another, often encountering a range of conditions, such as varying pH (Chung, K. et al., *Science* 243:192-197 (1989)). Intracellular pH in the various compartments can vary from as low as 4.5 to greater than 7.5. Insulin, for example, can be stored in soluble form in acidic secretory granules (PH 4.5-5.5) (Randall, L.. et al., *Ann. Rev. Microbiol.* 41:507-541 (1987)) but has limited solubility in bacterial cytoplasm (pH above 7) (Dinnbier, U. et al., *Arch. Microbiol.* 150:348-357 (1988)). Integral membrane proteins are typically insoluble in compartments lacking cellular phospholipids (Racker, E., *Fed. Proc.* 42:2899-2909 (1983)).

Secreted proteins require other proteins to maintain them in an unfolded conformation necessary for transport. It appears that certain mammalian proteins have evolved to function

as aids in such transport by modulating solubility of the transported proteins ("chaperonins," described below). Most secreted and membrane proteins have hydrophobic, N-terminal signal sequences that are recognized by an RNA-protein complex called the signal recognition particle (SRP). Purified SRP added to nascent proteins during translation prevents precursor exoproteins from folding into a conformation which will prevent subsequent translocation. Post-translationally, SRP has no effect unless the preprotein has been denatured in 8M urea, indicating a lack of "unfoldase" activity (Meyer, D.I., *Trends in Biochem. Sci.* 13:471-474 (1988)).

Ellis and colleagues (Ellis, J., *Nature* 328:378-379 (1987); Hemmingsen, S.M. et al., *Nature* 333:330-334 (1988); Ellis, J. et al., *Trends in Biochem. Sci.* 14:339-342 (1989)) recently used the term "molecular chaperon" to describe a ubiquitous class of proteins which function in ensuring that the folding of certain other polypeptide chains occurs correctly. "Chaperonins" refer to a class of toroidal, Mg^{++}ATP-dependent, chaperon proteins that specifically interact with the polypeptide chains to induce correct folding. Interaction with such proteins would prevent formation of incorrect structures which lack or have reduced biological activity and/or solubility (see below). Chaperonins have been described from bacterial (GroEL), mitochondrial (cpn60) and plant sources (Rubisco Subunit Binding Protein), e.g. as described herein.

Chaperons have also been shown to play a role in assisting protein assembly, disassembly and translocation across membranes (Hemmingsen et al., *Nature* 333:330-334 (1988); Cheng et al., *Nature* 337:620-625 (1989); Goloubinoff et al., *Nature* 337:44-47 (1989A,B); Kang et al., *Nature* 345:137-142 (1990); Vogel et al., *J. Cell Biol.* 110:1885-1895 (1990). Heat shock proteins (Hsp's), a class of proteins whose expression increases in cells subjected to thermal or other stresses, were studied intensively for many years, but only recently have they and their cognates been recognized as polypeptide binding proteins and putative chaperons (Gething et al. *Cell* 46:939-950 (1986); Pelham, *Cell* 46: 959-961 (1986); Ellis, *Nature* 328:378-379

(1987); Cheng et al., *Nature* 337:620-625 (1989), *Nature* 346:455-458 (1990); Ostermann et al., *Nature* 341:125-130 (1989).

In prokaryote (GroEL) (Hendrix, *J. Mol. Biol.* 129:375-398 (1979); Hohn et al., *J. Mol. Biol.* 129:359-373 (1979); Bochkareva et al., *Nature* 336:254-257 (1988); Goloubinoff et al., *Nature* 337:44-47 and *Nature* 342:884-889 (1989), mitochondria (cpn60) (Ostermann et al., *Nature* 341:125-130 (1989); Miller et al., *J. Mol. Biol.* 214:407-422 (1990); Viitanen et al., *J. Biol. Chem.* 267: 695-699 (1992) and chloroplast (Ribulose Biphosphate Carboxylase (RuBisCo) subunit binding protein) (Ellis, *Science* 250:954-959 (1990), chaperonins consist of either single or double toroids with seven-fold rotational symmetry, in conjunction with the co-chaperonin cpn 10, can correctly fold a number of prokaryotic proteins.

Heat shock proteins (HSPs) and their homolog, which are expressed either constitutively or in response to various stresses to a cell, are thought to belong to this functional category of molecular chaperons. The HSPs are a group of proteins induced when cells are stressed by shifts in temperature, exposure to toxins, contact with organic solvents, and the like. HSPs have recently been found to function in the unfolding of protein aggregates. Examples of HSPs include hsp70 hsc70 (Lewis, M.J. et al., *EMBO J.* 4:3137-3143 (1985)).

Horwich, A.R. et al., PCT Publication WO9004604 (5/3/90) discloses expression vectors and hosts containing the hsp60 protein or analogues thereof, which are useful for expressing the hsp60 protein.

Thus, in summary, the advantages of producing soluble and biologically active eukaryotic proteins, in particular mammalian and especially human proteins, in bacterial expression systems, as well as yeast or mammalian cells, are clear. The difficulties caused by the insolubility and/or lack of biological activity of these proteins, and the need for improved ways of producing such proteins in soluble form are well recognized (see Schein et al., *supra*). However, to date, adequate solutions to this problem have not been forthcoming. This major deficiency in the art is solved for the first time by the invention

disclosed herein.

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

One object of the present invention is to overcome one or more deficiencies of the related art.

Another object of the present invention is to provide a novel protein folding complex, or polypeptide subunits thereof, isolatable from eukaryotes, which can be used for renaturing or folding of proteins or polypeptides which lack, or have reduced, biological activity and/or solubility. Such folding may be used for co-expression, in bacterial or eukaryotic hosts, of heterologous proteins or polypeptides in a form having increased solubility and/or biological activity. Additionally, modified eukaryotic hosts may be provided according to the present invention, wherein the genes encoding polypeptide subunits of endogenous eukaryotic folding complexes can be introduced into the chromosomes of such eukaryotic host cells and amplified in conjunction with sequences encoding dihydrofolate reductase (*dhfr*) or adenosine deaminase that duplicate adjacent genes in response to exposure to inducing drugs, such as methotrexate or 2'-deoxycoformycin (dCF), respectively.

Such modified cells can be transformed with nucleic acid encoding a heterologous protein or polypeptide coupled to sequences encoding *dhfr*, and then induced using a suitable drug, such as methotrexate or dCF, respectively. Such amplification of the endogenous folding complex provides an alternative method according to the present invention for expressing heterologous polypeptides having increased solubility and/or biological activity due to interactions with the increased levels of endogenous folding complexes, optionally along with at least one of ATP, divalent cations and releasing factors, functionally

analogous to GroES releasing factor for the GroEL chaperonin in prokaryote.

This discovery provides a solution to a major problem plaguing the production of heterologous proteins in bacterial or eukaryotic hosts, in partially or substantially soluble form and with appropriate biological activity. Another object of the present invention is to provide eukaryotic host cells which are genetically modified to have inducible enhanced expression of endogenous folding complexes, wherein such cells can be used as hosts for expression. These obstacles are well-recognized in the art and have heretofore had no satisfactory solution.

Still another object of the present invention is to provide a recombinant nucleic acid useful in a bacterial or eukaryotic host for expressing at least one heterologous polypeptide in a form having increased solubility and/or biological activity due to association with a folding complex or subunit thereof of the present invention. Such a nucleic acid comprises at least one nucleotide sequence encoding at least one subunit derived from a eukaryotic folding complex, and at least one polynucleotide sequence encoding at least one heterologous polypeptide, the recombinant nucleic acid further including regulatory sequences for providing expression in a bacterial or eukaryotic host of both at least one polypeptide subunit of a folding complex and at least one heterologous polypeptide.

The above recombinant nucleic acids may be provided as expression vectors, preferably plasmid.

The present invention also provides a method of producing a heterologous polypeptide having increased biological activity or solubility in a bacterial or eukaryotic host, comprising the steps of: (a) providing the above recombinant nucleic acid in the host in a manner such that at least one polypeptide subunit of a folding complex encoded by at least one nucleotide sequence and a heterologous polypeptide encoded by the second nucleotide sequence can be expressed by the bacterial host; and (b) culturing the host under conditions conducive to the expression of the folding complex and the target protein or polypeptide in a form having increased solubility and biological

activity relative to expression of the heterologous polypeptide in the absence of the folding complex or subunits thereof.

The above method may further comprise the step of: (c) recovering the polypeptide expressed in step (b).

Preferably, in the above method, the polypeptide is one which, under culturing conditions, is produced in partially or substantially insoluble form in the absence of expression of the folding complex. In general, such a method is particularly useful where the heterologous polypeptide is one which would be irreversibly denatured if treated with a strong chaotropic agent.

The present invention is also directed to a bacterial or eukaryotic host capable of expressing a heterologous polypeptide having increased biological activity and/or solubility due to association with the folding complex, comprising a bacterial or eukaryotic cell transformed with at least one nucleotide sequence encoding at least one subunit of a eukaryotic folding complex, operably linked to an expression control region capable of controlling expression in a bacterial or eukaryotic host, and optionally further transformed with a polynucleotide encoding a heterologous protein or polypeptide operably linked to an expression control region capable of controlling expression of a heterologous protein or polypeptide in a bacterial or eukaryotic host, or progeny of the transformed cell. The above nucleotide sequences may be on the same or different nucleic acids or plasmid.

The present invention is also directed to a eukaryotic host transformed with any of the recombinant nucleic acids described herein, the host being capable of expressing the target polypeptide in a form having increased solubility and/or biological activity. A preferred bacterial host is *E. coli*, yeast or mammalian cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E present actin folding in rabbit reticulocyte lysate. Figure 1A shows an analysis on an 8.5% SDS polyacrylamide gel of total proteins from uninduced (-) and induced (+) host E. coli cells harboring expression plasmid

encoding a full length chicken β-actin cDNA. Figure 1B shows an autoradiograph of a 10.5% SDS polyacrylamide gel of total insoluble proteins recovered from a culture of ^{35}S-methionine-labelled host E. coli cells expressing chicken β-actin. In Figures 1A and 1B, the position of molecular wight markers is shown at the left. Figure 1C shows an autoradiograph of products of folding assays analyzed by autoradiography of a 4.5% non-denaturing polyacrylamide gel. Lane 1 of Figure 1C shows folding in buffer alone (0.1M KCl, 2mM $MgCl_2$, 1mM EDTA, 1mM DTT, 10mM Tris pH 7.2); lane 2 of Figure 1C shows folding in undiluted rabbit reticulocyte lysate; lanes 3-6 of Figure 1C shows lysate diluted with 2, 5, 10 and 20 volumes of buffer respectively. Figure 1D shows an autoradiograph of purified depolymerized mouse bran actin (20μg) run on a 4.5% non-denaturing polyacrylamide gel and stained with Coomassie blue. Figure 1E shows an autoradiograph of products of folding reactions done with lysate that had been either treated by heating to 56° for 45 mins (lane 1), by addition of ATP-γ-S to 2mM (lane 2), or by addition of EDTA to 5mM (lane 3). Analysis was on the same 4.5% non-denaturing gel shown in Figure 1C.

Figure 2 presents ability of folding complex-folded β-actin to copolymerize with purified actin. Samples taken at different stages during successive rounds of polymerization/depolymerization were analyzed on an 8.5% SDS Polyacrylamide gel. Lanes marked (-) show a control experiment in which the initial folding reaction was done in the absence of folding; lanes marked (+) show a parallel experiment in which the initial folding reaction was done in the presence of the partially purified folding (described in Figure 3a, lane 4, below). "St", starting material; "S_1" and "S_2", supernatant after one and two cycles of polymerization/depolymerization; "P_2", polymerized actin after two rounds of polymerization/depolymerization. Position of marker proteins (in kDa) is shown at the left.

Figures 3A-3F present purification of actin folding activity from rabbit reticulocyte lysate. Figure 3A shows non-denaturing polyacrylamide gel analysis of folding assays done on

fractions eluting from a MonoQ ion exchange column in the range 80-135mM $MgCl_2$. The band corresponding to correctly folded actin is arrowed. Figure 3B shows non-denaturing polyacrylamide gel analysis of folding assays done on starting material from the MonoQ column (lane 1), non-binding proteins (lane 2) and fractions eluted from the ATP agarose column with MgATP (lane 3-8). Figure 3C shows absorption profile of pooled fractions eluted from a ATP agarose column applied to a SUPEROSE 6 gel filtration column. Arrows show the position of marker proteins (thyroglobulin (670kDa), ferritin (440kDa), β-amylase (200kDa), bovine serum albumin (67kDa)) run under identical conditions. Figure 3D shows analysis on a 7% SDS polyacrylamide gel of fractions eluting from the Superose 6 column; molecular weight markers are at the left. Figure 3E shows non-denaturing gel analyses of folding assays done on fractions eluting from the Superose 6 column. Figure 3F shows, at lane 1, analysis on a non-denaturing polyacrylamide gel of material contained in the first peak emerging from the Superose 6 column and, at lane 2, analysis on a 7% SDS polyacrylamide gel of the Coomassie stained band shown in lane 1.

Figure 4 presents electron microscopic analysis of β-actin folding complex complexes. Samples from Superose 6 column fractions showing maximal β-actin folding activity (Fig. 3F) were examined either in the absence (panel a) or presence (panel b) of 0.1mM ATP. Bar=10nm.

Figures 5A-5C present kinetic analysis of β-actin folding. Figure 5A shows a non-denaturing gel analysis of a β-actin folding reaction done in the absence of ATP using partially purified folding complex (as presented, Figure 3a, lane 4). Figure 5B shows a profile of reaction products obtained in Figure 5A fractionated on a Superose 6 gel filtration column. Figure 5C shows an analysis on a 4.5% non-denaturing polyacrylamide gel of the peak fraction shown in Figure 5B, following incubation with 1mM ATP for the times (in minutes as indicated.

Figures 6A-6C: 6A and 6B, respectively, show one and two-dimensional analyses of purified β-actin-folding complex

folding. Figure 6C shows a western blot of an SDS polyacrylamide gel identical to that shown in Figure 6A, when probed with a monoclonal antibody to mouse TCP-1 (Willison et al., *Cell*, 57:621-632, 1989), wherein Lane 1 shows partially purified TCP-1 complex from mouse testis; lane 2; purified β-actin folding complex folding. The position of molecular weight markers (in kDa) is shown at the left of Figures 6A-6C.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has now been discovered that a eukaryotic folding complex, or one or more polypeptide subunits thereof, can be isolated, cloned and/or modified to provide folding of a partially or substantially insoluble target protein or polypeptide, such that the folding complex acts to fold the target protein or polypeptide in an alternative three-dimensional configuration which has increased solubility and/or biological activity relative to the form of the protein or polypeptide which is not folded by the folding complex.

Accordingly, a folding complex of the present invention can be used to renature or to correctly fold a partially or substantially insoluble or biological inactive form of a protein or polypeptide into a more soluble or active form having increased solubility and/or biological activity, *in vitro*, *in situ* or *in vivo*.

Renaturing or folding of a partially or substantially insoluble target protein or polypeptide according to a method of the present invention may comprise providing a denatured or unfolded target protein or polypeptide and renaturing the protein or polypeptide in the presence of a folding complex or subunit(s) thereof, of the present invention. In the context of the present invention, "renaturing" refers to re-folding a protein or polypeptide after it has been denatured by any known method. The term "folding" refers to folding a protein when it has not been denatured by a known method, but instead is correctly folded after translation in a host cell. The folding or refolding of a protein or polypeptide may occur under any known physiological conditions.

Alternatively or additionally, renaturing or folding a partially or substantially insoluble or inactive target protein or polypeptide according to a method of the present invention comprises co-expressing at least one subunit of a eukaryotic folding complex with at least one heterologous protein or polypeptide in a bacterial or eukaryotic host.

The present invention also includes eukaryotic cells that have been modified to have enhanced expression of endogenous eukaryotic folding complexes, which modified eukaryotic cells, such as yeast or mammalian cells can be used to express recombinant polypeptides, folded by the enhanced endogenous folding complexes, having increased solubility or biological activity.

The present invention is thus directed in one aspect to novel eukaryotic folding complexes, or one or more subunits thereof, and recombinant nucleic acids, as DNA or RNA, encoding therefor, which folding complex or subunits thereof may be used in a method for renaturing or folding a denatured or partially unfolded or substantially insoluble or biological inactive target protein or polypeptides into soluble forms having increased solubility and/or biological activity.

By the term "folding complex or "eukaryotic folding complex" is intended a multi-subunit protein which functions in protein folding, post-translational modification and/or transport to provide increased solubility and/or biological activity of a target protein or polypeptide relative to renaturing or folding in the absence of the folding complex or subunit. The presence of a folding complex during synthesis, post-translational modification, or transport results in increased biological activity and/or solubility of a renatured or translated target protein or polypeptide, by modification of folding, such as by a one or multi-step folding reaction. The folding reaction allows for formation of a folding complex-target protein or target polypeptide complex, followed by an ATP dependent or independent release, optionally with the interaction of one or more releasing factors, of the target protein or polypeptide in a native form having increased solubility and biological activity

due to interaction with the folding complex.

By the term "target protein" or "target polypeptide"is intended any known protein or polypeptide which is susceptible to renaturing or folding by a eukaryotic folding complex or polypeptide of the present invention. Such a target protein or polypeptide may be in denatured form due to chemical or physical known denaturing treatment, or in a form as translated in a host cell before the ultimate folding of the protein occurs.

According to the present invention, a eukaryotic folding complex includes an association of two or more polypeptide subunits such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15, up to 5-15 subunits preferably 8-9 subunits. Polypeptide subunits of a folding complex of the present invention have at least 80% homology, such as 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 and 99% homology, with subunits as described herein, which each have a molecular weight of about 55-62kDa, as presented in Examples I-IV. Such subunits of a eukaryotic folding complex of the present invention provide the biological activity of folding of a denatured protein in solution, as a release factor- or NTP-dependent or independent activity, such that a binary complex is formed between the folding complex and the denatured target protein, followed by dissociation and renaturing of the protein to form a renatured or correctly folded protein or polypeptide having increased solubility and biological activity over an alternative form of the protein folded in the absence of a folding complex or subunit according to the present invention.

Such subunits may include at least one subunit comprising one or more of the following amino acid sequences from the following Group A -

(A-1) AspGlyAsnValLeuLeuHisGluMetGlnPheGlnHis
(A-2) AlaAspLenValIleSerGlueGly

Group B

(B-1) AlaLeuGluIleIleProArg
(B-2) ArgSerLeuHisAspAlaIleMetIleValArg
(B-3) GlnLeuCysAspAsnAlaGlyPheAspAlaThrAsnIleLeuAsnLys

Group C

(C-1) GluNetHsnProAlaLew
(C-2) PheSerLenThrProGluLys

Group D

(D-1) AsnValLeuLeuAspProGlnLeuValProGlyGlyGlyAlaSer GluMetAla

Group E

(E-1) LysGlnMetGlnValLeuHisProAlaAlaArgHetLeuValGlu LeuSerLysLysvalIleAspProAlaThrAlaThrSerValAspXXX ArgAspIleLys

(E-2) LysAlaValAlaMetGluSerValAlaLys

(E-3) LysIleLeuIleAlaAsnThrGlyMetAspThrAsp

(E-4) IlePheGlySerArgValArgValArgValAspSerThrAlaLys

(E-5) MetIleGlnAspGlyLysGlyAspValThrIleThrAsnAspGlyAla ThrIleLeuLys

(E-6) GlyIleHisProThrIleIleSerGluSerPheGlnLys

(E-7) LysLeuGlyGlyThrIleAspAspXaaGluLeuValGluGlyLeuVal LeuThrGlnLys

(E-8) AlaGlyAlaAspGluGluArgAlaGluThrAlaArgLeuSerSerPhe

Group F

(F-1) PheAlaGluAlaPheGluAlaIleProArg

(F-2) GlnProGlySerGluAlaPheLeuAlaLys

A eukaryotic folding complex according to the present invention can be isolated according to the procedures exemplified in Examples 1 and Example 2, below, or similar modified known techniques, such that one of ordinary skill can routinely isolate a folding complex of the present invention without undue experimentation using the teaching and guidance presented herein.

Briefly, to isolate a subunit of a eukaryotic folding complex, a detectably labeled, such as radiolabeled, biologically active protein, such as actin, is denatured with a concomitant loss of biological activity, and a cell extract containing a putative eukaryotic folding complex is added to the denatured, labeled protein as a radiolabeled probe and mixture, with or without an NTP and divalent cation present, such as ATP and Mg++. The following labels for the active protein may be used.

By radioactively labeling the protein, or peptide probes, antibodies or antibody fragments thereto, it is possible to detect the labeled protein through the use of autoradiography or by a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S., et al., North Holland Publishing Company, New York (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by T. Chard, incorporated entirely by reference herein. The radioactive isotope can be detected by such means as the use of

a gamma counter or a liquid scintillation counter or by autoradiography.

The inactive protein can also be detectably labeled by providing peptide probes or antibodies to the inactive protein and linking the peptide probes or antibodies to an enzyme and use in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by calorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

It is also possible to label the inactive protein or a peptide probe or antibody thereto, with a fluorescent compound. When the fluorescently labeled peptide or antibody is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o- phthaldehyde and fluorescamine.

The inactive protein or a peptide probe or antibody thereto can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the inactive protein or peptide probe or anti-protein antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The inactive protein, or peptide probe or antibody thereto, also may be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged inactive protein, or peptide probe or antibody thereto, is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminal, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the inactive protein, or peptide probe or antibody thereto, of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic peptide probe or anti-target protein antibody increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent inactive protein, or peptide probe or antibody thereto, is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

The labeled denatured and inactive protein is thus used in folding assays to determine which fractions of a cell extract contain eukaryotic folding complex activity, as shown by active labeled protein, which also bind the labeled protein. Accordingly, fractions of cell extracts containing folding complex biological activity, as indicated by the presence of a biologically active labeled protein, such as labeled alpha or beta actin, wherein an actin polymerization/depolymerization assay may be used in conjunction with the labeled protein probe to determine extracts having folding complex activity.

Such cell extracts having folding complex activity are further purified by affinity chromatography, such as NTP agarose (as presented in Figure 3B), such as ATP, TTP, UTP, GTP or CTP, and by sizing on a Superose 6 gel filtration column.

As a non-limiting example, rabbit reticulocyte lysate may be used as a source of folding complex in combination with denatured radiolabeled beta-actin as the denatured inactive protein, followed by use of a beta-actin

polymerization/depolymerization assay, and labeled fractions containing folding actin activity are further purified by affinity chromatography on ATP agarose, followed by sizing purification on a Superose 6 gel filtration column.

As a non-limiting example of a folding complex obtained by such a method of the present invention, a molecular mass of the purified folding complex using actin from the Superose 6 column, showed a molecular mass of 670kDa, and a sedimentation co-efficient showing a molecular mass of about 800kDa. Additionally, SDS-PAGE analysis of the active folding complex peak from Superose 6 showed a cluster of 5 closely migrating bands having each a molecular weight of about 55-62kDa. Accordingly, the migrating bands suggest a multi-subunit folding complex having from 5-14 subunits, preferably 8-9 subunits. Each of the subunits has been purified in a sequencible form and can be sequenced according to known method steps, without undue experimentation.

Based on the amino acid sequence of one or more subunits of this eukaryotic folding complex, oligonucleotides can be labeled to isolate cDNAs encoding folding complex subunits from eukaryotic cells, such as mammalian or yeast cells, according to known method steps (see Ausubel et al. supra and Sambrook et al. supra). Examples of such subunit polypeptides are one or more in combination of groups A-F, or presented herein as A-1 to F-2.

For example, a nucleic acid encoding at least one subunit of a eukaryotic folding complex can be probed using a set of redundant oligonucleotide probes which encode an amino acid sequence of 4 or more amino acids of a subunit of a folding complex as described and isolated in the examples below, according to known method steps. See, e.g., Ausubel et al., eds. Current Protocols in Molecular Biology, Wiley Interscience, N.Y. (1987, 1992); and Sambrook et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989). Accordingly, a eukaryotic folding complex subunit has at least 80% amino acid homology to a subunit of the eukaryotic folding complex describes in Examples I-V below.

The target protein or polypeptide may be in the form of a denatured or translated heterologous polypeptide *in vitro, in situ or in vivo*. By the term "heterologous", as used herein, is intended a polypeptide not naturally expressed in the host, such as a polypeptide from a species other than the bacterial or eukaryotic species or cell in which the expression is occurring, for example, the recombinant host may have a recombinant nucleic acid encoding the target protein or polypeptide which has been introduced in expressible form, or into a parent host, such that the target protein or polypeptide is expressed by the recombinant host in recoverable amounts. Thus, as a non-limiting example in a particular expression system, a polypeptide of eukaryotic origin, or even of prokaryotic origin but not native to the host or host cell, is considered heterologous.

A nucleic acid according to the present invention may preferably encode at least one folding complex, a subunit or a set of subunits, such that a functional folding complex is produced, preferably having between 3 and 20 subunits, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 subunits, each subunit which may be about 50 to 65 kDa molecular weight such as 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65 kDa, or any range thereof. By the term "analog" of a eukaryotic folding complex or subunit is intended any combination of folding subunits that provide folding complex activity of denatured or expressed proteins. A protein or polypeptide molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in one molecule has statistically significant homology to the other (see, e.g., von Heijne, G., Sequence Analysis in Molecular Biology, Academic Press, New York, 1987)). A nucleic acid molecule encoding a "substantially similar" protein or polypeptide to a eukaryotic folding complex of the present invention will result in bacterial or eukaryotic host expression of a protein or polypeptide having similar protein renaturing or folding activity. For the purposes of the present invention, the requisite biological activity of a eukaryotic folding complex or folding complex analog is promoting the bacterial or eukaryotic expression of a

heterologous protein in a form having increased solubility or biological activity relative to the heterologous protein expressed without association or interaction with the folding complex or analog thereof. Alternative folding complexes or subunits, or analogues thereof, of the present invention can be used *in vitro, in situ or in vivo* to renature denatured proteins.

For example, folding complexes produced by bacteria could be purified and used *in vitro* to renature proteins by providing physiological type conditions, which would include the appropriate pH, ionic conditions, and the like, and which may optionally include the presence of ATP, divalent actions and/or protein release factors, as described herein.

Provided that two molecules possess a similar activity, folding complexes or heterologous proteins are considered analogues as that term is used herein even if one of the constituent molecules contain additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical.

The term "co-expression", as used herein, refers to expression in a host of both (a) a eukaryotic folding complex, analog or subunit thereof, and (b) a heterologous protein or polypeptide of interest, and is intended to encompass: (1) concomitant expression of both one or more subunit polypeptides of a folding complex and at least one heterologous protein driven by a single inducible promoter and contained on a single plasmid, i.e. polycistronic expression; (2) concomitant expression of both folding complex proteins and heterologous protein driven by separate inducible promoters contained on a single plasmid; (3) concomitant expression of such proteins encoded by nucleic acid sequence contained in separate plasmid; (4) sequential expression of the folding complex subunits and the heterologous proteins driven by separate promoters, on a single or on separate plasmid, by first inducing the promoter driving expression of the folding complex or subunits thereof, followed by inducing the promoter driving expression of the protein or polypeptide of interest; or (5) expression of the folding complex or subunits thereof and at least one heterologous protein or polypeptide of interest as a

fusion protein.

The preferred mode of co-expression involves identical but separate promoters for each of the folding complex or subunits, and the heterologous protein polypeptide of interest, and concomitant induction of multiple identical promoters to co-express both the folding complex or subunits thereof and at least one target heterologous protein or polypeptide, to obtain the target protein or polypeptide of interest in a form having increased solubility and/or biological activity relative to folding or renaturing of the target protein or polypeptide in the absence of the folding complex or subunits thereof, such as having partial or substantial solubility.

Recombinant DNA and/or RNA molecules as nucleic acids of the present invention, can be produced through any of a variety of techniques, preferably by application of known recombinant DNA or RNA techniques. Techniques for synthesizing such molecules are disclosed by, for example, Wu, R., et al. (Prog. Nucl. Acid. Res. Molec. Biol. 21:101-141 (1978), hereby incorporated entirely by reference). Procedures for constructing recombinant molecules in accordance with the above-described method are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989) and Ausubel et al., eds., Current Protocols in Molecular Biology, Wiley Interscience, NY (1987, 1992) which references are entirely herein incorporated by reference.

A nucleotide sequence encoding one or more folding subunits and a polynucleotide encoding a target heterologous polypeptide sequence to be expressed in soluble form according to the present invention, are recombined with vector DNA in accordance with conventional techniques.

Such techniques employ blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, phosphatase treatment to avoid undesirable joining, ligation with appropriate ligase, or the synthesis of fragments by the polymerase chain reaction (PCR). According to known methods,

see, e.g., Ausubel, et al. supra., and Sambrook et al., supra.

Intact native proteins can be made in bacteria by providing a strong, regulated promoter and an efficient ribosome binding site. To express a prokaryotic gene that has a strong ribosome binding site, only an appropriate inducible prokaryotic or bacteriophage promoter may need to be used. To express a eukaryotic gene, both an inducible prokaryotic promoter and a ribosome binding site must be provided. Levels of expression may vary from less than 1% to more than 30% of total cell protein.

A nucleic acid, such as DNA or RNA, is said to be "capable of expressing" a polypeptide or protein or "expressible" if it contains nucleotide sequences which contain signals for transcriptional and translational initiation. Such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the signals for transcriptional and/or translational initiation and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression of the nucleic acid encoding the polypeptide or protein. The precise nature of the signals required for gene expression vary from organism to organism, but shall in general include a promoter region which contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

Techniques for preparing DNA molecules having the appropriate sites required for efficient expression of proteins are disclosed by Sambrook et al., supra, and are well known in the art.

Eukaryotic genes and prokaryotic genes with weak ribosome binding sites require that an efficient site be provided, using methods disclosed in Sambrook et al.. Other ribosome binding sites are disclosed, for example, by Gold, L., et al. (Ann. Rev. Microbiol. 35:365-404 (1981)).

One skilled in the art will know when and how to create the necessary restriction sites for insertion of the DNA or the

cDNA encoding the polypeptide of interest without undue experimentation. A polylinker, well-known in the art (see Sambrook et al., *supra*), will provide the terminus for ligation of the 3' end of the DNA sequence encoding the polypeptide to be expressed.

In other embodiments of the present invention, the nucleic acid sequence encoding the polypeptide of interest, including a stop codon, is incorporated in the vector at the cloning site described above. The nucleic acid encoding folding complex subunits, in a non-limiting example, is present along with a single open reading frame encoding a protein of interest such as t-PA, Factor VIII, Colony stimulating factors, interleukins, insulin, growth factors (e.g., TGFs, FGFs, PDGF, etc.) or any known protein expressible in a host cell. As stated above, the cloning site into which the DNA sequence encoding the polypeptide(s) of interest has been inserted is adjacent to DNA encoding a ribosome binding site. The restriction enzyme recognition sequence engineered into the vector DNA or into the cDNA encoding the polypeptide of interest provides the ATG initiation codon in the proper location to initiate expression of the polypeptide.

The promoter sequences useful in the present invention may be of either prokaryotic cell or bacteriophage origin for bacterial hosts or eukaryotic origin for eukaryotic hosts. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Strong promoters are most preferred, including those which recognize the T3, SP6 and T7 polymerase, the P_L promoter of bacteriophage lambda and the recA promoter. The most preferred promoter is one which is capable of recognizing the T7 polymerase. The sequences of such polymerase recognition sequences are disclosed by Watson, J.D. et al. (In: Molecular Biology of the Gene, Fourth Edition, Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA, (1987)).

Examples of other suitable promoters include promoters capable of recognizing the polymerase of T4 (Malik, S. et al., J. Biol. Chem. 263:1174-1181 (1984)), T3 (Bailey, J.N. et al., Proc. Natl. Acad. Sci. (U.S.A.) 80:2814-2818 (1983)) and Sp6

(Davanloo, P. et al., Proc. Natl. Acad. Sci. (U.S.A.) 81:2035-2039 (1984)); the P_R and P_L promoters of bacteriophage lambda (Lambda II, Hendrix, R.W., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1980)); the trp, recA, heat shock, and lacZ promoters of E. coli; the α-amylase (Ulmanen, I., et al., J. Bacteriol. 162:176-182 (1985)) and the σ-28-specific promoters of B. subtilis (Gilman, M.Z., et al., Gene 32:11-20 (1984)); the promoters of the bacteriophages of Bacillus (Gryczan, T.J., In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY (1982)); Streptomyces promoters (Ward, J.M., et al., Mol. Gen. Genet. 203:468-478 (1986)); the int promoter of bacteriophage lambda; the bla promoter of the ß-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pPR325, etc. Prokaryotic promoters are reviewed by Glick, B.R., (J. Ind. Microbiol. 1:277-282 (1987)); Cenatiempo, Y. (Biochimie 68:505-516 (1986)); Watson, J.D. et al. (In: Molecular Biology of the Gene, Fourth Edition, Benjamin Cummins, Menlo Park, CA (1987)); and Gottesman, S. (Ann. Rev. Genet. 18:415-442 (1984)). All of the above listed references are incorporated by reference herein.

Preferably, the introduced nucleotide sequences will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Such a vector is said to include a "replicon," or a DNA sequence capable of directing autonomous replication and maintenance of the vector DNA extrachromosomally in a prokaryotic cell. Alternatively, the introduced DNA sequences may be integrated into the host chromosome, for example, by lysogenization with a bacteriophage. Construction of such bacteriophage, e.g., lambda for *E. coli*, P22 for *Salmonella typhimurium*, and the like) by introducing the DNA sequence or sequences of interest, for example, from a plasmid, is well-known in the art (see Sambrook et al., *supra*; Hendrix, R.W., *supra*).

Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from

those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Useful prokaryotic vectors which are commercially available from vendors such as BioRad Laboratories (Richmond, CA) or Pharmacia (Piscataway, NJ) include plasmid such as those capable of replication in E. coli, such as, for example, pBR322, pBR329, pUC plasmid (e.g., pUC8 and pUC9), ColE1, pSC101, pACYC 184, πVX, lambda ZAP, and vectors derived therefrom. For description of such plasmid, see Sambrook et al. (*supra*). Bacillus plasmid include pC194, pC221, pT127, etc. Such plasmid are disclosed by Gryczan, T. (In: The Molecular Biology of the Bacilli, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmid include pIJ101 (Kendall, K.J., et al., J. Bacteriol. 169:4177-4183 (1987)), and streptomyces bacteriophages such as ϕC31 (Chater, K.F., et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmid are reviewed by John, J.F., et al. (Rev. Infect. Dis. 8:693-704 (1986)), and Izaki, K. (Jpn. J. Bacteriol. 33:729-742 (1978)).

Preferred vectors according to the present invention are the plasmid of the pET series, disclosed by Studier et al. (*supra*) and shown in Table 1, below. Most preferred are the plasmid pET3a, pET3b, pET3c, pET11a, pET11b, and pET11c, which have a bla selective marker, a BglII upstream site, NdeI ATG cloning sites, and BamHI fusion cloning sites with 12 codons before the first in-frame codon. Additional information including expression signals and downstream elements are provided herein (Table I). Target DNAs are cloned into the vectors, preferably the pET vectors, by standard techniques (Sambrook et al., *supra*).

Preferred hosts for the present invention are bacteria or yeast. The most preferred bacterial hosts are E. coli, preferably recA mutants of E. coli (to prevent recombination within the plasmid DNA sequences). In other embodiments, other bacterial species can be used, for example Bacillus, Salmonella,

Pseudomonas and Streptomyces species. See, e.g., Ausubel et al, *supra*; Sambrook et al *supra*, as available, e.g., from the ATCC (Rockville, Maryland).

Other suitable host cells in this context may include bacteria or eukaryotic cells such as yeast, insect, mammalian and human cells. Suitable host cells of the present invention may include microorganisms, e.g., of the genera *Aeromonas, Aspergillus, Bacillus, Escherichia, Kluyveromyces, Pichia, Rhodococcus, Saccharomyces and Streptomyces*.

Host cells comprising a nucleic acid which encodes a heterologous target protein or polypeptide or folding complex or subunit of the present invention may be grown under conditions that provide expression of a desired polypeptide in recoverable or commercially useful amounts. See, e.g., Ausubel, *supra*, at §§ 1 and 13.

According to a preferred aspect of the present invention, the discovery of eukaryotic folding complexes can be used as a basis for techniques to amplify the expression of endogenous folding complexes in eukaryotic host cells used to express heterologous proteins or polypeptides having increased biological activity and/or solubility.

Using oligonucleotides probes, as described herein, which are specific for DNA encoding subunits of folding complexes, loci on the chromosomes of eukaryotic host cells can be identified according to known method steps, e.g., using probes corresponding to a portion of at least 8 amino acids of a folding complex subunit as described in Examples I-V, below. Once the chromosomal loci of one or more folding complex subunits has been determined, known method steps can be used to amplify expression using inducible genes that cause chromosomal gene amplification, such as the *dhfr* gene or the adenosine desminase gene (ADA). The induced amplification of folding complex loci is expected to provide modified eukaryotic host cells that express increased levels of recombinant proteins have increased biological activity and/or solubility, due to enhanced expression of one or more endogenous folding complex subunits. Accordingly the present invention encompasses all such modified eukaryotic host cells.

See, e.g., Sambrook et al, *supra*, §§ 16.9-16.15 and 16.28 and all references cited herein, which references are incorporated entirely herein by reference.

For introducing a nucleic acid of the present invention into a bacterial cell, known procedures may be used according to the present invention such as by transfection, e.g., using calcium phosphate precipitation, electroporation, DEAE dextran, pelletizing with a DNA gun or using a recombinant phage virus. See, e.g., Ausubel, *supra*, and Sambrook et al. Other known procedures may also be employed to obtain a recombinant host cell that expresses a heterologous target or folding complex according to the present invention, as will be apparent to those skilled in the art.

Non-limiting examples of yeast hosts generally available for use according to the present invention, are yeast expression systems, such as those employing Saccharomyces cerevisiae and Pichia pastoria respectively. See, for example, U.S. patents No. 4,456,082 and No. 4,837,147 (Saccharomyces expression), Nos. 4,855,231, 4,808,537 and 4,857,467 (Pichia and No. 4,806,472 and No. 4,859,596 Kluyveromyces expression), which references are herein incorporated herein by reference.

The choice of an appropriate yeast strain for transformation will be determined in large part by the selectable marker(s) and other features of the vector employed. Yeast strains which are exemplary of those suitable for used according to the present invention are strain, with genotype (available from the Yeast Genetic Stock Center, Berkeley, California); strain ATCC 52683, with genotype a his2 ade1 trp1 met14 ura3 (aka "strain IL166-5B," also available for the American Type Culture Collection.

For introducing a nucleic acid of the present invention into a yeast cell, the most commonly used protocol, the lithium acetate method, exploits the fact that alkali cations make yeast cell membrane permeable to DNA; in addition, uptake of foreign DNA is promoted by the presence in the medium of a high-molecular-weight molecule, polyethylene glycol. An alternative method, spheroplast transformation, may be used but is more time-

consuming than the lithium acetate procedure though it results in a higher efficiency of transformation per input DNA. See, e.g., Ausubel, *supra*, at section 13.

For baculoviral expression systems, conventional transformation procedures with pACRP-derived vectors are used to transform suitable host cells including those of, e.g., *Spodoptera* (such as sf9 cells), *Trichoplusia*, and *Heliothis*. See Luckow & Summers, Biotechnology 6:47-55 (1988); Miller, Ann. Rev. Microbiol. 42:177-199 (1988); Maeda, Ann. Rev. of Microbiol. 34:351-72 (1989). For vaccinia viral expression systems, see Chakrabarti et al., Molec. Cell. Biol. 5:3401-9 (1985) and Mackett et al., J. Virol. 49:857-864 (1984). See also Ausubel, *supra*, section 16.8 - 16.11, and U.S. patent 5,077,214, the entire contents of which are herein incorporated by reference.

Another type of expression system entails the use of mammalian host cells transformed with a nucleic acid within the present invention as described herein, as known in the art available, e.g., from the ATCC (Rockville, Maryland). For both yeast and mammalian expression systems, there are conventional transformation and screening protocols which may be employed pursuant to the present invention. Standard methodology in this regard is detailed in Ausubel et al, *supra*, and Sambrook et al, *supra*.

Methods of the present invention may be directed to the use of the nucleic acids, vectors and hosts described above to express heterologous proteins in bacterial or eukaryotic cells in a form having increased solubility and/or biological activity. Such methods include, for example, culturing transformed bacterial or yeast host cells, and obtaining from them, either secreted into the medium or via extraction procedures well-known in the art, the expressed heterologous polypeptides. Since such proteins may interact with the co-expressed folding complex, it may be useful to use antibodies specific for the folding complex, or specific for the protein of interest, for conventional isolation and purification procedures. Such antibodies can be polyclonal or monoclonal.

Additionally, eukaryotic release factors may need to

be present for proper folding via a folding complex of the present invention. A non-limiting example of such a release factor is for the folding of t-PA; as presented in Example V below, two releasing factors are a 200 KDa releasing complex and a 55 KDa protein which release factors do not bind ATP.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLE I: Assay for Biological Activity of Eukaryotic Folding complex.

Radiolabeled actin was obtained by expressing a full length cDNA encoding chicken β-actin (Cleveland et al. Cell 20:95-105 1980) inserted into the pET3d vector (Studier et al. Meth. Enzymol. 185 60-89 1990). A 2 ml culture of E. coli BL21DE3 cells harboring the recombinant plasmid was induced with IPTG and incubated for 2 hours in the present of 0.2 mg/ml rifampicin and 1mCi of the mixture of ^{35}S-methionine and ^{35}S-cysteine (^{35}S-Express, New England Nuclear). Labelled cells were harvested by centrifugation, resuspended in 50 μl of 10 mM Tris-HCl, pH7.2, 1mM EDTA and lysed by incubation with lysozyme (0.1 mg/ml). $MgCl_2$ was added to 10 mM and DNaseI to 10μ/ml, and the lysate left on ice for 2 mn. Triton X-100 was added to 1% and the lysate centrifuged at 4° for 5 mins at 15,000 x g. The pellet was dissolved by persistent vortexing in freshly made 7M guanidine HCl, 20 mM Tris HCL pH7.2, 10mM DTT, cleared by centrifugation at 15,000 x g for 5 mins at room temperature, and the supernatant applied to a 0.5 x 17 cm column of Sephadex G50 equilibrated in 7.5M urea (freshly prepared and deionized by stirring with Amberlite mixed be resin) contain 10 mM Tris-HCl, pH7.2 and 2 mM DTT. The radioactivity emerging in the void volume (typically 1-2 x 10^6 cpm/μl) was used as a probe in folding assays in which aliquots of probe were diluted 100-fold. The reaction was allowed to proceed at 30° for 45 mins; products were analyzed on 4.5% non-denaturing polyacrylamide gels containing 80mM MES, pH6.85, 1mM $MgCl_2$, 1mM EGTA, 1mM ATP. The

running buffer was identical in composition to the gel buffer except that the ATP concentration was 0.1 mM. In some experiments (i.e. kinetics analyses), ATP was omitted and the gels were prerun for 1 hr at 90V with buffer containing 1mM glutathione. Gels were run for 1.5-2 hours at 90V, stained with Coomassie blue, destained and fluorographed.

EXAMPLE II: Use of Folding Assay to Isolate Eukaryotic Folding complex

A full length cloned cDNA encoding chicken β-actin (Cleveland et al. Cell 20:95-105, 1980) was inserted into the pET3d vector and expressed as a ^{35}S-methionine-labelled peptide in E. coli under conditions where host protein synthesis is suppressed by rifampicin (Studier et al. Meth. Enzymol. 185:60-89 1990). This resulted in the accumulation of a large quantity of actin (Figure 1A), most of which was insoluble. The insoluble bacterial products were dissolved in 7M guanidine HCl, and passed through a column a Sephadex G50 in 7.5M urea. When analyzed on an SDS polyacrylamide gel, about 50% of the radioactivity emerging in the void volume from this column was found and migrate as intact actin; the remainder was contained in two minor bands of lower molecular weight and a faint background smear of degraded polypeptides (Figure 1B). This material, which had a specific radioactivity of about 3 x 10^6cpm/μg, was used as a probe in folding assays.

These assays were done by diluting the probe 100-fold into rabbit reticulocyte lysate; among a variety of cell and tissue extracts, we found that this material was most active in terms of its ability to fold the denatured probe. Following incubation at 30° for 45 min, the reaction products were analyzed on a 4.5% non-denaturing gel polyacrylamide gel (Figure 1c). In control reactions in which the probe was diluted into buffer alone, all the radioactivity remained at the origin (lane 1). Lanes 2-6 show the products of reactions in which the labelled denatured probe was added to successive dilutions of reticulocyte lysate. In each case, three species enter the non-denaturing gel: a slow migrating band that remains close to the origin; a

middle band; and a fast migrating band that appears in decreasing yield with increasing dilution of the lysate. The fast migrating band comigrated with active monomeric actin purified from brain and run on a 4.5 non-denaturing gel (Figure 1D). From these data and from experiments discussed below the production of properly folded β-actin (Figure 2 and Table 1), was demonstrated, such that this band represented correctly folded β-actin.

The folded actin generated in the dilution assays could represent molecules that had interacted with one or more folding complexes, or by a spontaneous folding reaction occurring in the very high concentration of protein present in the reticulocyte extract. The reaction was found to be folding complex mediated, because activity was lost upon exposure to heat and was dependent on the presence of Mg++ and ATP (Goloubinoff et al. *Nature* 337:44-47 (1989a); Gething et al. *Nature:* 355:33-45 (1992). Lane 1 in Figure 1E shows the products of a dilution assay done with lysate pretreated by heating to 56° for 45 mins; and no materials comigrating with active actin is visible. Similarly, inclusion of either 2mM ATP-γ-S in the reaction or sufficient EDTA to chelate all Mg++ ions abolished the conversion of the denatured probe into correctly folded actin (Figure 1E, lanes 2 and 3).

Example III: Electron Microscopy for Purified Eukaryotic Folding complex

Samples in Superose 6 column buffer with or without added ATP were absorbed onto glow-discharge activated carbon coated grids and negative stained with uranyl acetate.

We examined the purified actin folding complex fraction from Superose 6 by electron microscopy. These experiments revealed particles having a toroidal structure with an outer diameter of 16nm and an inner diameter of 6 nm (Figure 4A). Significantly, no structures identifiable as side views were seen. This is in contrast to GroEL, which has similar molecular mass but displays both end views and side views when prepared for electron microscopy in an identical manner, the side views being rectangular structures with four striations (Hendrix, *J. Mol. Biol.*, 129:375-398, 1979; Hohn et al., *J. Mol. Bio.*, 129:359-373, 1979; McMullen and Hallberg, *Md. Cell Biol.*, 7:4414-4424, 1988;

Hutchison et al., *EMBO, J.*, 8:1484-1490, 1989). Presumably, proteins present in the β-actin folding complex are arranged such that, for reasons of shape, charge distribution, or both, the complexes fall on the electron microscope grid in a unique orientation.

Based on its sedimentation coefficient, the cytoplasmic folding complex would be expected to contain about 5-14 subunits of polypeptides, each having an average MW of at least 55-62 kDa. Complexes of folding complex of the present invention have little rotational symmetry, thus differing from the seven-fold rotationally symmetric end views of GroEL and cpn 60 (compared in Viitanen et al. *J. Biol. Chem.* 110:1885-1895 1990) as well as the eight-fold rotationally symmetric end views of the folding complex-like complexes isolated from archaebacteria (Phipps et al. *EMBO J.* 10:1711-1722 (1991); Trent et al. *Nature* 354:490-493 (1992)).

The addition of 0.1mM ATP to the folding complex induced a dramatic change in its appearance: a large majority of the particles appeared as 16 nm circles with two prominent striations across the central portion (Figure 4B). Some of these complexes were elongated, with an oval or rectangular appearance that is similar to side views of GroEL and to the striated structures seen in micrographs of complexes isolated archaebacteria. In micrographs of GroEL and archaebacterial folding complex, however, the striated complexes occur together with toroidal view seen in roughly equal proportions, while the striated complexes of folding complex of the present invention constitute the vast majority of structures seen in preparation with Mg-ATP. Since the predominance of either the striated form or the toroidal form of the acting folding complex is correlated with the presence or absence of Mg-ATP, the two forms reflect two distinct conformations of folding complexes of the present invention.

Two models are expected to explain these observations. First, the toroidal and striated structures may be alternative conformations, both seen from the same end-on view. If this is the case, side views of the complexes are not present. An

alternative possibility, suggested by the resemblance of the toroidal and striated complexes to the end and side views of GroEL, is that the electron micrographs show two different views of the particle: end views in the absence of Mg-ATP and side vies in its presence. In this scenario, ATP is expected to induce a change in the shape or charge distribution of the complexes, altering the way that they adsorb onto the electron microscope grid. Such a conformational change would have to be dramatic to account for the observed preponderance of one form or the other, particularly since the number of particles absorbed per grid square is not appreciably affected by Mg-ATP.

Kinetics of Folding: Formation of a Binary Complex and ATP-Dependent Release

As shown above, the β-actin folding experiments using partially purified folding complex yielded two products that enter non-denaturing gels: a slow and a fast migrating band (e.g., Figure 3B). The slow migrating and the fast migrating band is a product of the folding reaction band is an binary dependent in the folding reaction, as shown by kinetic relationship between the two products. In the absence of ATP, a β-actin folding experiment gave rise to the slowly migrating band upon non-denaturing gel electrophoresis, with no detectable production of the fast-migrating native actin band (Figure 5A). When the products of this reaction were applied to a Superose 6 gel filtration column, more than 50% of the radioactivity emerged as a single peak in the range 600-800kDa (Figure 5B), which is close to the mass expected of a binary complex between β-actin and a eukaryotic folding complex. Upon incubation of the peak fractions with 1mM ATP, there was a progressive decline in radioactivity contained in this binary complex with time, and a corresponding increase in radioactivity in the fast migrating band that comigrates with monomeric β-actin (Figure 5C). Since virtually all the input radioactivity in this experiment was contained in the binary complex, these data show that the conversion of this complex to correctly folded β-actin in an ATP-dependent manner. Accordingly, the folding reaction by a eukaryotic folding complex of the present invention can occur in

one embodiment as a two-step process: (1) an NTP-, such as ATP-, independent association of unfolded polypeptide with folding complex, and (2) an NTP-, such as ATP-, dependent release of correctly folded product. Other NTPs include GTP, UTP, TTP and CTP.

Example IV: Analysis of Folded β-Actin By a Eukaryotic Folding complex of the Present Invention

Two tests were performed to show that correctly folded β-actin was produced in these assays. First, the ability of the reaction products to copolymerize with native cytoplasmic actin was measured as follows.

Copolymerization with brain actin

About 0.5mg of purified mouse brain actin (Levilliers et al., 1984) suspended in buffer A (described in Spudich and Watt, 1971) was added to the products of a folding reaction done with partially purified folding complex (Figure 3a, lane 4). KCL, $MgCl_2$ and ATP were added to 50mM, 2mM, and 1mM, respectively and the mixture was incubated at 4° for 2hr. Polymerized actin was recovered by centrifugation at 100,000 x g for 1 hr; the pellet was resuspended at a concentration of 0.6 - 0.8 mg/ml and dialyzed overnight at 4° in buffer A. This material was clarified by centrifugation at 4° for 20 minutes at 45,000 xg. Actin present in the supernatant was then polymerized by adjusting the final conditions to 50 mM KCl, 2mM $MgCl_2$ and 1mM ATP, incubating at 4° for 2 hr and centrifuging as described above. Subsequent cycles of depolymerization and repolymerization were repeated in the same manner.

In a control folding reaction, done in the absence of folding complex, no radioactive material was detectable after two cycles of polymerization/depolymerization (Figure 2, lane P_2 (-)). In contrast, in an experiment in which the denatured probe was folded in a folding complex mediated reaction, labeled β-actin cycled efficiently with carrier brain actin through two successive rounds of polymerization and depolymerization (Figure 2, lane P_2 (+)).

b) Binding to Sepharose-linked DNAseI

Second, the affinity of *in vitro* folded B-actin for DNaseI (see Lazarides and Lindberg, *Proc. Natl. Acad. Sci. USA* 71:4742-4746 (1974), was tested a property that has been used for the purification and characterization of native actin (Korn, *Proc. Nat'l. Acad. Sci. USA* 71:4742-4746 1982).

The products of a folding complex-mediated β-actin folding reaction were taken through two cycles of polymerization/depolymerization with mouse brain actin as described above. This material was applied to a DNaseI-Sepharose column prepared and run as described by Lazarides and Lindberg (1974).

In this experiment, the starting material consisted of the products of a folding complex mediated folding reaction that had been taken through two successive cycles of polymerization and depolymerization with added carrier brain actin. All of the labelled β-actin contained in the starting material bound to and eluted from the DNaseI column with the same efficiency as the carrier brain actin (Table 2).

TABLE 1

	Starting material	Non-binding	Bound and eluted
Total cpm (x 10^{-4})	21.6	7.5	8.1
Total protein (μg)	132	45.2	52
Specific activity (cpm/μg)	1640	1660	1560

The efficiency with which the folding reaction produced coassembly-competent β-actin was estimated from the specific activity of the β-actin during the polymerization/depolymerization cycles. After the first cycle, the specific activity was 50% of the starting specific activity, decreasing to 30% after the second third cycles. Assuming that the starting material was about 50% radiochemically pure intact β-actin (Figures 1B and 2), the yield of correctly folded β-actin

was about 60%. The efficiency of the folding reaction was also measured by comparing the yield of correctly folded product (excised from non-denaturing gels) with the input radioactivity. By this criteria, the efficiency of conversion was in the range 45%-55%. The concentration of folding complex is not expected to be a limiting factor in folding reactions done using undiluted reticulocyte lysate, since experiments using less probe did not result in any detectable increase in the efficiency of folding.

In vitro dilution reactions were used as an assay to purify the β-actin folding activity from reticulocyte lysate. Folding assays were performed on material eluted from a MonoQ ion exchange column with a gradient of $MgCl_2$ (Figure 3a). About 90% of the actin folding activity (as judged by the presence of the fastest migrating band on a non-denaturing gel) eluted at 90-100mM $MgCl_2$

Based on the co-appearance of the slowest migrating band and correctly folded actin at this and all subsequent purification steps, the slow-migrating band represented a binary complex consisting of actin bound to folding complex subunits, as further shown by kinetic experiments suggesting a precursor-product relationship between the two, as presented.

We further purified the folding complex activity by affinity chromatography on ATP agarose (Figure 3B) and by sizing on a Superose 6 gel filtration column. In each case, the location of actin folding complex was monitored by folding assays and non-denaturing gel analysis of the reaction products (Figure 3B and 3E). A single symmetrical peak corresponding to active folding complex emerged from the Superose 6 column at the same position as thyroglobulin, which has a molecular mass of 670 kDa. On an isokinetic sucrose gradient, the same material sedimented as a sharp band at 19S, slightly slower than GroEL, which sediments at 20S and was used as a reference. Based on its sedimentation coefficient relative to that of the 840kDa GroEL, we calculated a molecular mass of about 800kDa. The lower estimate based on migration through the Superose 6 column was expected to be due to weak interactions with the Superose matrix.

When the active folding complex peak from Superose 6

was analyzed on an SDS polyacrylamide gel, a cluster of five very closely migrating bands in the molecular weight range 55-62kDa was observed, apparently in non-equimolar yield (Figure 3D). In contrast, when the same material was analyzed on a native polyacrylamide gel, a single sharp Coomassie-staining band was obtained (Figure 3F). Excision of this material and re-analysis under denaturing conditions yielded the same cluster of closely migrating polypeptides apparent upon direct analysis of the first peak emerging from the Superose 6 column (Figure 3D). These results suggested that the folding polypeptides were derived from a single multi-subunit complex.

Conclusion

While chaperonins in bacteria, mitochrondria and chloroplasts have been relatively well characterized, the molecules that mediate protein folding in the eukaryotic cytoplasm have thus far proved elusive. Examples I-IV above show that a multimolecular complex purified from a eukaryotic cell lysate correctly folds a eukaryotic protein. This complex exhibits chaperonin-like properties in that it has a torodial structure that forms a binary complex with unfolded β-actin, and releases the native product in an ATP-dependent manner.

The chaperonins from bacteria, mitochondria, and chloroplasts are each assembled from a single protein. In contrast, a eukaryotic folding complex of the present invention consists of at least five resolvable polypeptides by analysis on SDS polyacrylamide gels and at least seven distinct species by two-dimensional gel analysis (Figure 6A, B). Accordingly it is expected that the eukaryotic folding complex of the present invention consists of a multisubunit-protein assembled from similar but distinct polypeptides.

Folding complex subunits of the present invention may have some degree of homology but differ in their polypeptide composition.

The function of folding complexes is in one embodiment, to couple the hydrolysis of ATP to protein folding, presumably through a conformational change. A folding complex of the present invention thus is expected to provide at least two

distinct conformations of the folding complexes, and the folding reaction can be separated into at least two steps. These observations suggest that, in the absence of ATP, the folding complex complexes may be in one conformation that binds the unfolded or partially folded protein to form a relatively stable complex. Upon addition of ATP, the complex switches to another conformation and the peptide is released.

It has been shown for GroEL that folding occurs at the surface of the complex and requires hydrolysis of about 100 molecules of ATP (Martin et al., *Nature*, 352:36-42, 1991); folding may result from the step-wise release of different regions of the polypeptide chain, promoting ordered intrapeptide interactions (Rothman, *Cell*, 59:591-601, 1989). Given the overall similarity in shape and release kinetics among the known chaperonins, it seems likely that the folding reactions they catalyze share a common mechanism. However, the different folding complexes cannot always function interchangeably, e.g., purified mitochondrial cpn60 failed to fold or form a binary complex with the denatured β-actin probe.

EXAMPLE V: Folding Cytoplasmic Protein Unrelated to Actin by a Eukaryotic Folding Complex of the Present Invention

To determine whether a folding complex of the present invention folds other cytoplasmic proteins, two cytoskeletal proteins that are structurally unrelated to actin were tested. In assays similar to those used in actin folding experiments, the folding complex was found to efficiently form a binary complex with either fully denatured tubulin. It is therefore expected that a folding complex, or combination of subunits thereof, is capable of catalyzing the folding of a spectrum of cytoplasmic proteins or heterologous proteins *in vivo*, *in situ* and *in vitro*, according to the present invention.

EXAMPLE VI: Expression of Eukaryotic Folding Complex with a Heterologous Protein in E. Coli.

E. coli strains BL21(DE3) or HMS174(DE3) (Studier et al., *supra*) are used. Cells are grown in NZ broth.

The starting vectors used are pET3a, pET3b, pET3c, pET11a, pET11b and pET11c, described by Studier et al., *supra*.

DNA sequences encoding, a folding complex of the present invention, and a t-PA to be expressed, are removed from other plasmids and inserted into the pET plasmids using standard techniques, See Ausubel, *supra*, or Sambrook, *supra*.

The cDNA encoding t-PA is referenced in Hinnen et al., In: Yeast Engineering, Butterworths, pp. 193-213 (1990), and references cited therein; which are incorporated entirely herein by reference.

The newly constructed plasmids, containing folding complex subunit-encoding sequences and t-PA, are introduced into the bacterial hosts described above using standard techniques. Shine-Dalgarno sequences are introduced at the appropriate sites following amplification by PCR using synthetic oligonucleotides according to standard procedures, See, e.g., Ausubel, *supra;* and Sambrook, *supra*, entirely incorporated herein by reference.

Cells are grown until they reach a density of 0.8-1.0 Absorbance units (at 550 nm). The cells are then induced with 0.4 mM IPTG for 2 to 2.5 hours.

At the end of the induction period, the cells are lysed in a lysing buffer containing PIPES, MES, HEPES or TRIS at pH 6.7-7.5, 1 mM $MgCl_2$, 1 mM EGTA. Lysozyme is added (10 μg). The mixture is incubated at room temperature until sticky, and was then centrifuged at 52,000 x g in a Beckman TL-100 for 15 minutes. The supernatant, containing the soluble fraction of the cells is then taken for analysis.

SDS-PAGE is performed using an 8% Laemmli gel. Induced cultures are expected to show an increase in a band corresponding to t-PA.

A Western blot of the above gel is prepared, and probed with monoclonal antibodies (mAbs) specific for t-PA. Results are expected to show that only in the presence of the folding complex subunit genes are soluble t-PA produced by the appropriately transformed bacteria.

mAbs for t-PA are used described, e.g., by Blose et al., *J. Cell Biol.* 95:229a (1982), and are available from Amersham. The Western blot is developed using a sandwich assay, wherein the nitrocellulose containing the blotted material is

incubated with the anti-t-PA mAb, followed by rabbit anti-mouse immunoglobulin antibody and followed by ^{125}I-protein A. The blots are developed using standard conditions.

The soluble fraction of cells transformed with the plasmid containing folding complex and t-PA are purified according to the above examples and fractions obtained are run on SDS gels, Western blotted, and probed with anti-t-PA mAbs in a sandwich assay as described above. The results, are expected to show that the folding complex/t-PA complex is of high molecular weight.

Thus, by constructing a vector which expresses a folding complex derived from subunit polypeptides each drive by a T7 promoter, a eukaryotic protein is expressed having increased solubility and biological activity, relative to folding in the absence of the folding complex, which protein expected to be produced with good results. In the absence of the co-expression of the folding complex or subunits thereof, these proteins are expected to be expressed in a t-PA form, having reduced biological activity or solubility.

The soluble t-PA produced, as described above, is tested for its biological activity (in addition to ability to bind to anti-t-PA antibodies, as shown above). Radiolabeled soluble t-PA, produced in *E. coli* as described above, is tested for its known biological activity. It is expected that the soluble and biologically active t-PA produced in *E. coli*, or similarly in yeast or mammalian cells, are commercially useful, indicating that a eukaryotic protein produced according to the present invention has increased biological activity and/or solubility.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the generic concept of the present invention. Therefore, such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein.

WHAT IS CLAIMED IS:

1. A recombinant nucleic acid useful in the production of a bacterial or eukaryotic host capable of expressing at least one partially or substantially insoluble or biologically inactive heterologous target protein or polypeptide in form having increased solubility or biological activity, comprising

(a) at least one expressible nucleotide sequence encoding at least one subunit of a eukaryotic folding complex; and

(b) at least one polynucleotide encoding at least one of said heterologous target protein or polypeptide,

wherein association of said at least one subunit with said target protein or polypeptide, under physiological conditions, induces renaturing or folding of said heterologous protein or polypeptide in a form having increased solubility or biological activity relative to the solubility or biological activity of the heterologous protein or polypeptide which has been renatured or folded in the absence of said at least one subunit of said folding complex.

2. A recombinant nucleic acid according to claim 1, wherein said eukaryotic host is selected from a bacteria, a yeast or a mammalian cell.

3. A non-naturally occurring eukaryotic folding complex, useful for renaturing or folding a target protein or polypeptide in a form having increased solubility, said eukaryotic folding complex comprising

(a) at least one subunit consisting essentially of at least one polypeptide having at least 80% homology with a subunit of 55-62 kDa molecular weight on SDS-PAGE of a rabbit folding complex derived from rabbit reticulocyte lysate,

wherein association of said eukaryotic folding complex with the target protein or polypeptide in denatured or unfolded form under physiological conditions induces renaturing or folding of said target polypeptide in a form having significantly increased

solubility relative to the solubility of the target protein or polypeptide which has been renatured or folded in the absence of said folding complex.

4. A folding complex according to claim 3, wherein said eukaryotic folding complex comprises 5 to 15 of said subunits, each of said subunits having a molecular weight of 55-62 kDa under SDS-PAGE.

5. A folding complex according to claim 3, wherein said at least 80% homology is at least 90%.

6. A folding complex according to claim 3, wherein said folding complex is a toroid protein having 9 subunits.

7. A folding complex according to claim 6, wherein said eukaryotic folding complex has said subunit of 55-62 kDa molecular weight according to SDS-PAGE.

8. A method for renaturing or folding a target protein or polypeptide in an increased soluble or biologically active form having increased solubility or biological activity, comprising:

(a) providing said target protein or polypeptide in denatured form as a denatured target protein or polypeptide;

(b) contacting said denatured target protein or polypeptide with a folding complex according to claim 3, such that said folding complex interacts with said target protein or polypeptide to induce renaturation of said target protein or polypeptide in said increased soluble or biologically active form; and

(c) recovering said increased soluble or biologically active form of said target protein or polypeptide.

9. A method for producing a partially or substantially

insoluble or biologically inactive heterologous target protein or polypeptide in an increased soluble or biologically active form, comprising:

(a) providing a eukaryotic or bacterial host comprising a recombinant nucleic acid according to claim 1;

(b) culturing said host under culturing conditions such that said folding complex and said target protein or polypeptide are co-expressed in recoverable amounts, wherein the target protein or polypeptide interacts with the folding complex to release the target protein or polypeptide in said increased soluble or biologically active form relative to the solubility or biological activity of the target protein or polypeptide renatured or folded in the absence of the folding complex; and

(c) recovering the target protein or polypeptide in said increase soluble or biologically active form.

10. A method according to claim 9, further comprising

(d) purifying the target polypeptide recovered in step (c).

11. A method according to claim 9, wherein said host further comprises an amplification gene which is inducible by a drug to amplify said at least one expressible nucleotide sequence encoding at least one subunit of a eukaryotic folding complex, such that said host expresses said eukaryotic folding complex in at least three fold higher amounts in the presence of said drug than without said drug.

12. A method according to claim 9, wherein said amplification gene is selected from a *dhfr* gene and an adenosine deaminase gene, and said drug is selected from methotrexate or a derivative thereof, and 2'-deoxycoformycin, respectively.

13. A method according to claim 9, wherein said

interaction of said target protein or polypeptide with said folding complex is a two step reaction involving a binary complex of said folding complex and said target protein or polypeptide, optionally requiring the presence of at least one of a nucleoside triphosphate, a divalent cation and a protein releasing factor.

14. A method according to claim 13, wherein said nucleoside triphosphate is adenosine triphosphate and said divalent cation is Mg^{++}.

15. A recombinant bacterial or eukaryotic host capable of expressing a heterologous target protein/polypeptide in increased soluble form, comprising an expressible nucleic acid according to claim 1, or progeny of said recombinant host cell.

16. A bacterial or eukaryotic host according to claim 15, wherein said first and said second nucleotide sequences are in separate nucleic acids.

17. A bacterial or eukaryotic host capable of expressing a heterologous polypeptide in soluble form, comprising a bacterial or eukaryotic cell transformed with a recombinant nucleic acid according to claim 1, or progeny of said transformed cell.

18. A bacterial host capable of expressing a heterologous polypeptide in soluble form, comprising a bacterial or eukaryotic cell transformed with a recombinant nucleic acid according to claim 10, or progeny of said transformed cell.

19. A method for isolating a eukaryotic folding complex according to claim 3, comprising

(a) providing fractions of a cell extract containing a biologically active eukaryotic folding complex according to claim 3;

(b) contacting said fractions with a labeled, biologically active protein in substantially denatured form;

(c) recovering one or more of said fractions having labeled protein associated with other proteins as labeled fractions;

(d) assaying said labeled fractions for biological activity of said labeled protein to obtain active labeled fractions;

(e) performing affinity chromatography on said active labeled fractions using NTP-agarose to produce an affinity fraction; and

(f) performing gel filtration chromatography using a Superose 6 gel filtration column to obtain a gel fraction as a single symmetrical peak containing the folding complex.

20. A method according to claim 19, wherein said labeled, active protein is a labeled t-PA.

21. A method according to claim 20, wherein said detectable label is a radiolabel, an enzymatic label, a fluorescent label or a luminescent label.

22. A method according to claim 19, wherein said label is a sulfur radioisotope label.

23. A method according to claim 20, wherein said assaying in step (d) is a t-PA assay.

24. A method according to claim 19, wherein said NTP-agarose in said affinity step (e) is ATP-agarose.

25. A eukaryotic host, comprising

(i) a recombinant nucleic acid encoding said heterologous target protein or polypeptide; and

(ii) a recombinant polynucleotide incorporated into the chromosome of said host cell, said polynucleotide encoding an amplification gene which is inducible by a drug to amplify at least one chromosomal nucleotide sequence encoding at least one subunit of an endogenous folding complex.

26. A eukaryotic host according to claim 25, wherein said host is selected from a yeast, insect or mammalian cell.

27. A method for producing a partially or substantially insoluble or biologically active heterologous target protein or polypeptide in an increased soluble or biologically active form, comprising:

(a) providing a eukaryotic host according to claim 25;

(b) culturing said host under culturing conditions such that (i) said folding complex is expressed in enhanced amounts due to the presence of said drug and (ii) said target protein or polypeptide

is expressed in recoverable amounts, wherein the target protein or polypeptide interacts with the folding complex to release the target protein or polypeptide in said increased soluble or biologically active form relative to the solubility or biological activity of the target protein or polypeptide renatured or folded in the absence of the folding complex; and

(c) recovering the target protein or polypeptide in said increase soluble or biologically active form,

wherein said host expresses said eukaryotic folding complex in at least three fold higher amounts in the presence of said drug than without said drug.

28. A method according to claim 27, further comprising

(d) purifying the target protein or polypeptide recovered in step (c).

29. A method according to claim 27, wherein said amplification gene is selected from a *dhfr* gene or an adenosine deaminase gene, and wherein said drug is selected from methotrexate or a derivative thereof for inducing the *dhfr* gene, and 2'-deoxycoformycin for inducing the adenosine deaminase gene.

30. A method according to claim 27, wherein said interaction of said target protein or polypeptide with said folding complex is a two step reaction involving a binary complex of said folding complex and said target protein or polypeptide, optionally requiring the presence of at least one of a nucleoside triphosphate, a divalent cation and a protein releasing factor.

31. A method according to claim 30, wherein said nucleoside triphosphate is adenosine triphosphate and said divalent cation is Mg^{++}.

32. A folding complex according to claim 1, wherein said target protein or polypeptide is in a partially or substantially insoluble form.

33. A non-naturally occurring eukaryotic folding complex, useful for renaturing or folding a biologically inactive biological activity, said eukaryotic folding complex comprising

(a) at least one subunit consisting essentially of at lease one polypeptide having at least 80% homology with a subunit of 55-62 kDa molecular weight on SDS-PAGE of a rabbit folding complex derived from rabbit reticulocyte lysate,

wherein association of said eukaryotic folding complex with the target protein or polypeptide in denatured or unfolded form under physiological conditions induces renaturing or folding of said target polypeptide in a form having significantly increased biological activity relative to the biological activity of the target protein or polypeptide which has been renatured or folded in the absence of said folding complex.

34. A folding complex according to claim 32, wherein said eukaryotic folding complex comprises 5 to 15 subunits, each of said subunits having a molecular weight of 55-62 kDa under SDS-PAGE.

35. A folding complex according to claim 33, wherein said at least 80% homology is at least 90%.

36. A folding complex according to claim 33, wherein said folding complex is a toroid protein having 9 subunits.

37. A folding complex according to claim 35, wherein said eukaryotic folding complex has said subunit of 55-62 kDa molecular weight according to SDS-PAGE.

38. A folding complex according to claim 33, wherein said target protein or polypeptide is in a partially or substantially insoluble form.

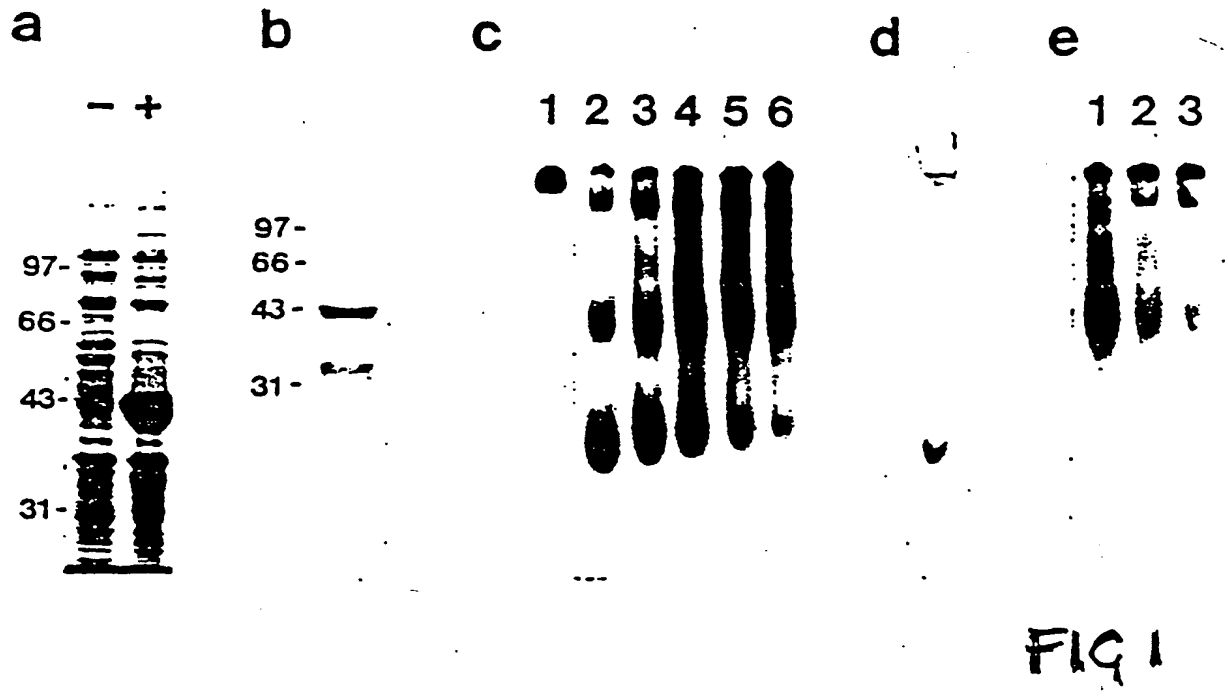

FIG 1

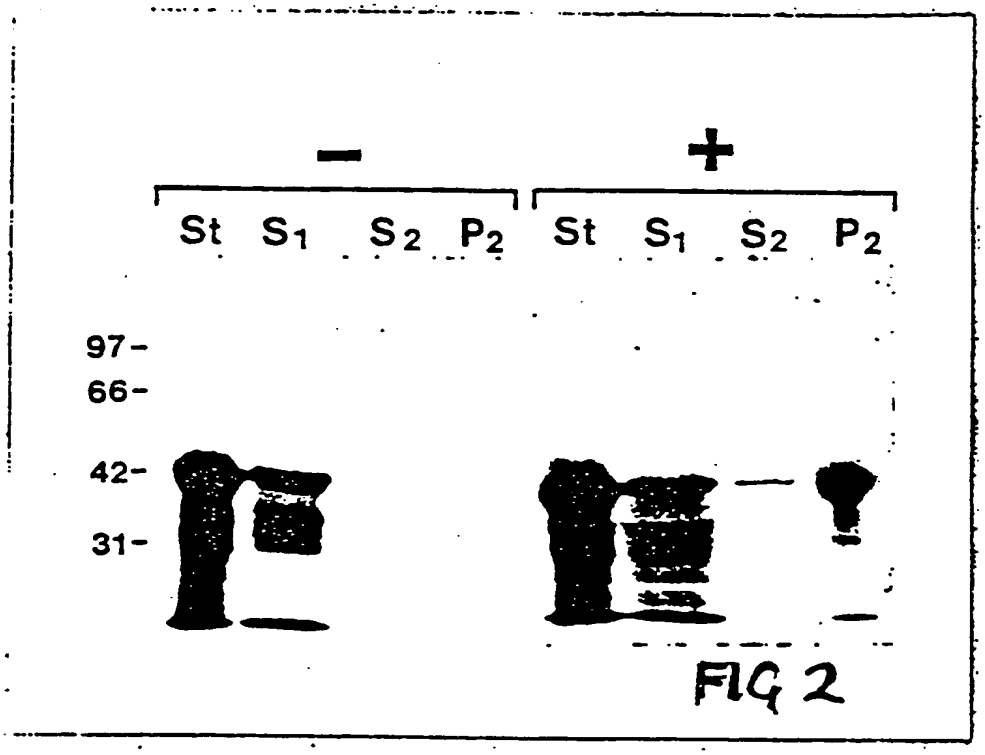

FIG 2

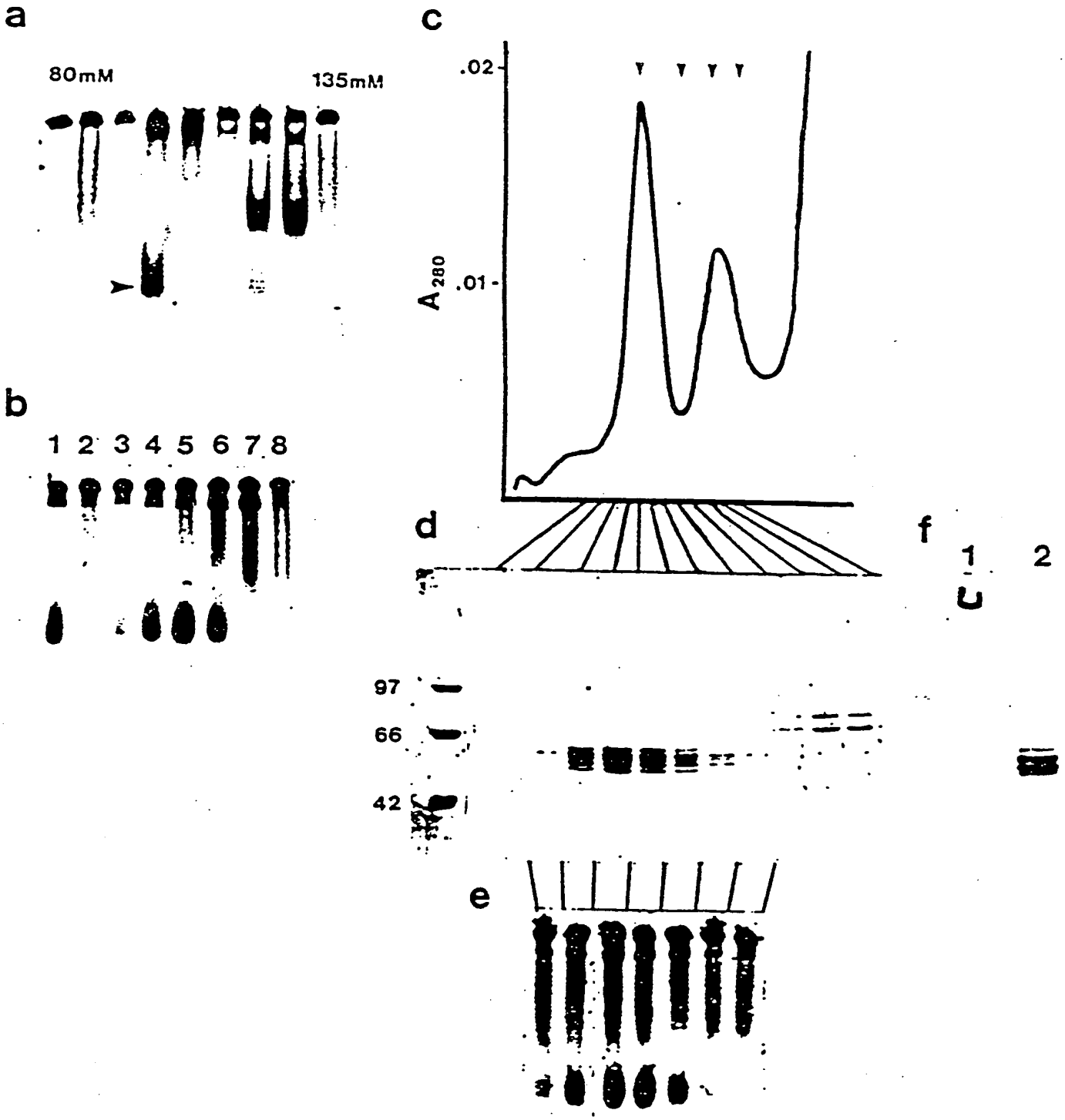

FIG 3

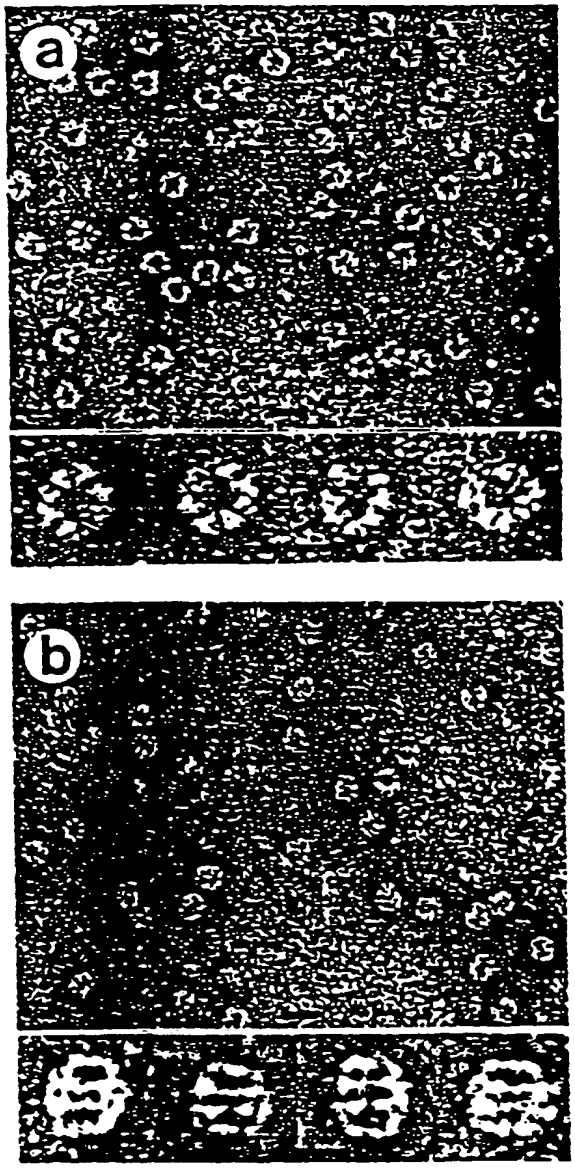

FIG 4

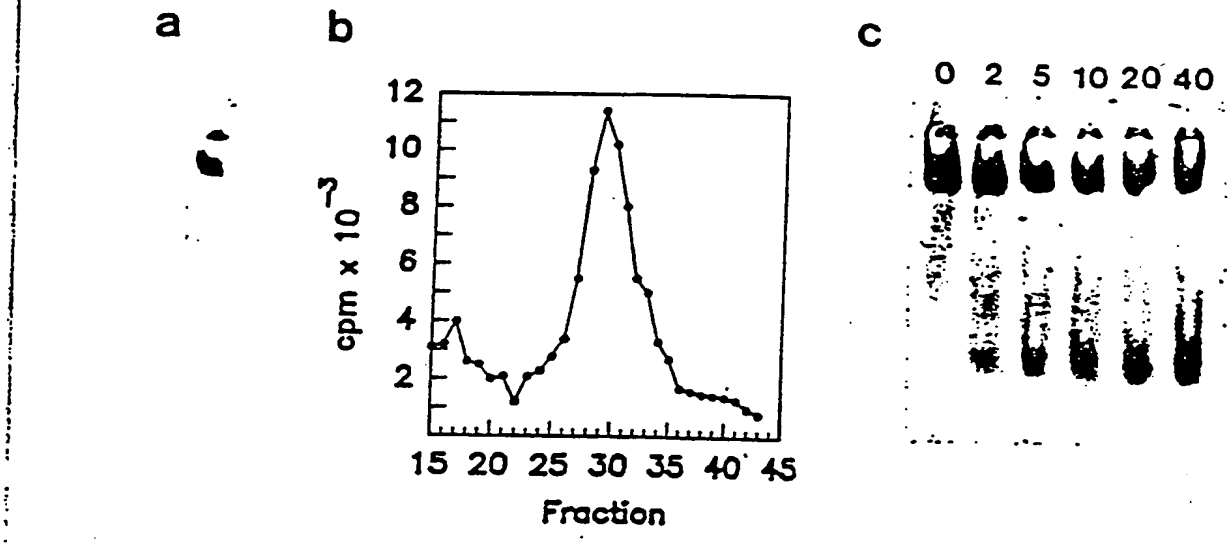

FIG 5

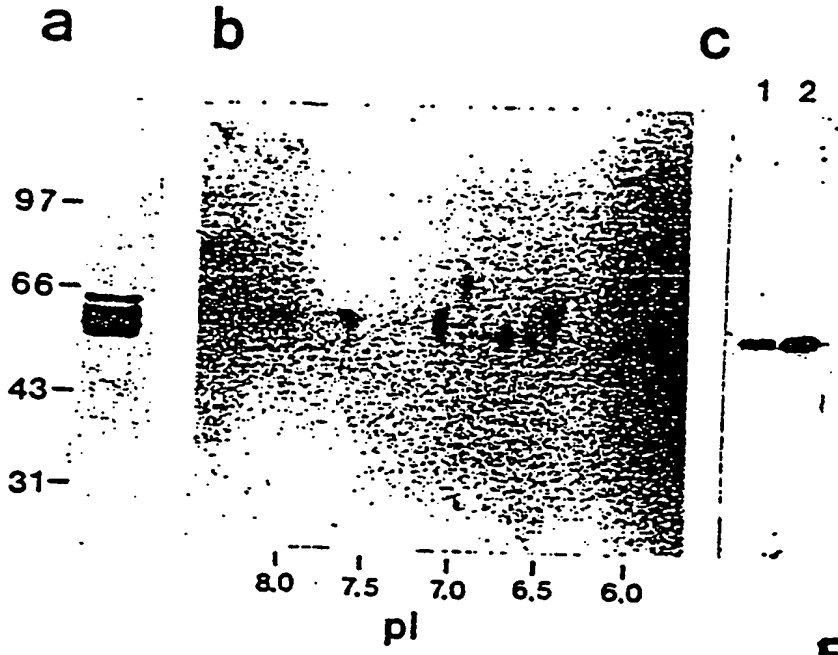

FIG. 6

NTERNATIONAL SEARCH RE[illegible]T

International Application No PCT/US 93/05568

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C12N15/67; C07K3/12; C07K15/00
C12P21/02; C12N15/70; C12N1/19; C12N1/21

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; C12P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NATURE vol. 337, 5 January 1989, MACMILLAN JOURNALS LTD., LONDON,UK; pages 44 - 47 P. GOLOUBINOFF ET AL. 'GroE heat-shock protein promote assembly of foreign procaryotic ribulose bisphosphate carboxylase oligomers in Escherichia coli' cited in the application see page 45, left column, line 1 - page 47, left column, line 17	1,2,8,9, 27
X	WO,A,9 004 604 (IOWA STATE UNIVERSITY RESEARCH FOUNDATION) 3 May 1990 cited in the application see page 34, line 29 - page 40, line 28	8

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 SEPTEMBER 1993	11 -10- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HORNIG H.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	NATURE vol. 342, 21 December 1989, MACMILLAN JOURNALS, LTD.,LONDON UK; pages 884 - 889 P. GOLOUBINOFF ET AL. 'Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP' cited in the application see page 885, left column, paragraph 2 - page 888, right column, paragraph 2	8
Y	NATURE vol. 354, 12 December 1991, MACMILLAN JOURNALS, LTD.,LONDON,UK; pages 490 - 493 J.D. TRENT ET AL. 'A molecular chaperone from a thermophilic archaebacterium is related to the eucaryotic protein t-complex polypeptide-1' cited in the application the whole document see figure 3	1,2
Y	SCIENCE vol. 250, 16 November 1990, AAAS, WASHINGTON,DC,US; pages 954 - 959 R.J. ELLIS 'Molecular chaperones: The plant connection' cited in the application see page 958, right column, line 4 - line 8	1,2
A	J. BIOL. CHEM. vol. 267, no. 2, 15 January 1992, AM. SOC. BIOCHEM.,BALTIMORE,US; pages 695 - 698 P.V. VIITANEN ET AL. 'Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring' cited in the application the whole document	1-38

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	TRENDS IN BIOTECHNOLOGY vol. 8, no. 12, December 1990, ELSEVIER SCIENCE PUBL.,AMSTERDAM,NL; pages 354 - 357 A.A. GATENBY ET AL. 'Chaperonin assisted polypeptide folding and assembly: implications for the production of functional proteins in bacteria' the whole document	1-38
P,X	CELL vol. 69, no. 6, 12 June 1992, CELL PRESS, CAMBRIDGE, MA,US; pages 1043 - 1050 Y. GAO ET AL. 'A cytoplasmic chaperonin that catalyzes beta-actin folding' see page 1043, right column, paragraph 2 - page 1047, right column, paragraph 1	3-8,19, 22,24, 33-38
P,X	NATURE vol. 358, 16 July 1992, MACMILLAN JOURNALS, LTD.,LONDON,UK; pages 245 - 248 M.B. YAFFE ET AL. 'TCP1 complex is a molecular chaperone in tubulin biogenesis' see page 245, left column, paragraph 1 - page 248, right column, paragraph 1	1-8,19, 22,24, 33-38
P,X	WO,A,9 311 248 (CIBA-GEIGY AG) 10 June 1993 see page 5, line 11 - page 18, line 13; claims 1-24	1,2, 8-10,27, 28
E	WO,A,9 313 200 (NOVO NORDISK) 8 July 1993 see page 5, line 4 - page 12, line 6; claims 1-34	1,2

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9305568
SA 76063

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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